hydrogen atoms for the group nearer to the center of the cavity. As discussed previously,[5] the O(4) oxygen atoms are hidden behind the C(3)-H and C(5)-H hydrogen bonds and cannot interact with substrate molecules.

Although the hexagon formed by the six O(4) atoms is rather regular, Figure 5 clearly indicates that the α-CD cavity is elliptical in shape; viz. at the O(6) side of the cavity, the shortest C(5)-H···C(5)-H distance is about 3.4 Å and the longest is 4.5 Å; at the O(2), O(3) side the shortest C(3)-H···C(3)-H distance is about 4.6 Å and the longest is 5.5 Å. The α-CD molecule has adapted its cavity somewhat to the dimensions of the enclosed methanol molecules.

Hydrogen Bonding and Packing Scheme. The hydrogen bonding scheme within the crystal structure is explained by Table IX and Figure 7 and shown in the stereoplot of Figure 6. As all the hydroxyl hydrogen atoms except those attached to disordered oxygen atoms were located, the assignment of hydrogen bonds is certain even in cases where rather long O···O distances are observed. Table IX shows that hydrogen bond lengths vary from 2.632 to 3.145 Å and O-H···O angles vary from 87 to 174°.

The packing of the α-CD molecules is described schematically in Figure 8. Since the void of an individual α-CD is blocked on both ends by adjacent, symmetry related α-CD molecules, this structure belongs to the cage type previously described in greater detail.[6,12,19] Channel type structures with the α-CD molecules stacked like coins in a roll have been observed in the adducts with salts like potassium acetate[20] and polyiodide[21] and with long, molecular guest molecules.[19]

Confirmation of Mechanism of α-CD Inclusion Formation. As mentioned at the outset, this x-ray structural study was undertaken in order to find out whether the α-CD molecule in the α-CD-methanol adduct would assume a strained, collapsed conformation as in the "empty" water adduct or an unstrained, open, cyclic conformation common to all the other thus far investigated adducts. From the above it is clear that the latter structure exists here. The α-CD macrocycle is rather cyclic and regular and all of the six O(2)···O(3) intramolecular hydrogen bonds have formed, in agreement with the proposed inclusion formation mechanism.

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Supplementary Material Available: Table III listing structure factors, 24 pages. Ordering information is given on any current masthead page.

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Malformin C, a New Metabolite of *Aspergillus niger*

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Abstract: A strain of *Aspergillus niger* collected from mold damaged rice produces malformin C, a new, highly toxic metabolite. It was established to be the disulfide of *cyclo*-D-cysteinyl-D-cysteinyl-L-valyl-D-leucyl-L-leucyl. Amino acid sequencing was accomplished using gas chromatographic mass spectrometry. A 100-m capillary gas chromatographic column allowed the separation of two diastereomeric *N*-trifluoroacetyl dipeptide methyl esters and the chirality of a critical L-valyl-D-leucyl dipeptide could be determined with the aid of this method.

As part of a continuing search for food borne mycotoxins we had occasion to investigate a highly toxic methylene chloride extract of an *Aspergillus niger* strain AN-1 that originally had been collected from mold-damaged rice. The physiologically active principle was separated from inactive metabolites by chromatographic techniques and obtained in the form of an amorphous powder, mp >300° dec. A high-resolution mass spectrum indicated a composition of $C_{23}H_{39}N_5O_5S_2$ and infrared absorptions at 3280 and 1630 cm^{-1} were attributed to NH and amide functions, respectively. A proton NMR spectrum revealed inter alia six methyl groups bound to carbon atoms, and deuterium exchange caused the disappearance of five NH signals. The toxin thus appeared to be a pentapeptide and the spectral data were in general agreement with those of the malformins, a family of cyclic pentapeptides discovered by Curtis[3] and found to produce malformations of bean plants

Table I. Amino Acid Residues in Malformin A_1, A_2, B_1, and B_2

A_1	Cys:Val:Leu:Ile	2:1:1:1
A_2	Cys:Val:Leu or Ile	2:2:1
B_1	Cys:Val:Leu:*a*Ile	2:1:1:1
B_2	Cys:Val:Leu	2:2:1

Table II. Ratio of Amino Acids in Malformin C Hydrolysates

	Roche[a]	MIT[b]
	Desthiomalformin C	
Val	1.00	1.00
Leu	1.78	1.84
Ile	0.01	0.03
Ala	1.86	1.55
Cys	0.0	0.0
	Malformin C	
Val	1.00	1.00
Leu	1.87	1.99
Ile	0.01	0.08
Cys	1.57	1.60

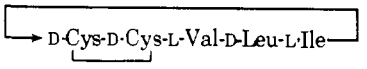
[a] We are grateful to Drs. H. Maehr and P. Scheidl, Hoffmann-La Roche Inc., Nutley, N.J., for those determinations. [b] These measurements were kindly performed by Mr. Douglas Faller, Department of Nutrition and Food Science, MIT.

Table III. Optical Rotations of Amino Acids Obtained by Hydrolysis of Desthiomalformin C

Val,	$[\alpha]^{26}D$ +22.1° (*c* 3.9, 6 N HCl); L-Val[a]
	$[\alpha]^{23}D$ +22.9° (*c* 0.8, 20% HCl)
Ala,	$[\alpha]^{26}D$ −10.1° (*c* 3.6, 6 N HCl); D-Ala[b]
	$[\alpha]^{25}D$ −13.6° (*c* 1,6 N HCl)
Leu,	$[\alpha]^{26}D$ +1.2° (*c* 4.3, 6 N HCl); L-Leu[c]
	$[\alpha]^{25}D$ +15.2° (*c* 2, 6 N HCl)

[a] Merck Index, 8th ed, Merck and Co. Rahway, N.J., 1968, p 1100. [b] M. S. Dunn, M. P. Stoddard, L. B. Rubin, and R. C. Bovie, *J. Biol. Chem.*, 151, 248 (1943). [c] M. P. Stoddard and M. S. Dunn, *ibid.*, 142, 336 (1942).

as well as curvatures of corn roots. The amino acid compositions of the four known malformins are tabulated in Table I. The most thoroughly investigated malformin A_1 was initially believed to be the disulfide form of *cyclo*-L-isoleucyl-D-cysteinyl-L-valyl-D-cysteinyl-D-leucyl[4] but in two more recent reports Bodanszky proposed structure **1** which was verified by synthesis.[5,6]



1

Reduction of our toxin with dithiothreitol in liquid ammonia followed by in situ benzylation of the resulting dithiol yielded a dibenzylmalformein $C_{37}H_{53}N_5O_5S_2$. Desulfurization with Raney nickel in refluxing methyl cellosolve afforded the corresponding desthiomalformin $C_{23}H_{41}N_5O_5$. Comparison of the ratios of amino acids in hydrolysates of known malformins with those derived from our toxin indicated the presence of a new compound which we named malformin C. Hydrolyses of desthiomalformin C and malformin C on a preparative scale gave results summarized in Tables II and III. Malformin C thus contains L-valine, D-cysteine, D-leucine, and L-leucine in a ratio of 1:2:1:1.

For amino acid sequencing dibenzylmalformein C was partially hydrolyzed and the oligopeptides were transformed to the *N*-trifluoroacetyl methyl esters. Reduction with lithium aluminum deuteride was then followed by conversion of the polyamino alcohols to the *O*-trimethylsilyl derivatives.[7] Using a gas chromatography–mass spectrometer–computer system eight peptide derivatives were identified (Figure 1) and these can be assembled only as shown in Table IV.

Desthiomalformin C was then submitted to the same analytical technique and the 16 peptide derivatives (Figure 2) identified lead to the unique sequence shown in Table V.

Table IV. Oligopeptide Fragments Identified (see Figure 1) and Reassembled Sequence of Dibenzylmalformein C

Scan	
111	Cys-Val
148	Cys-Val-Leu
023	Val-Leu
075	Val-Leu-Leu
032	Leu-Leu
152	Leu-Leu-Cys
116	Leu-Cys
187	Cys-Cys
	Cys-Val-Leu-Leu-Cys

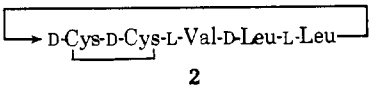
Table V. Oligopeptide Fragments Identified (see Figure 2) and Reassembled Sequence of Desthiomalformin C

Scan	
044	Ala-Val
101	Ala-Val-Leu
144	Ala-Val-Leu-Leu
171	Ala-Val-Leu-Leu-Ala
064	Val-Leu
116	Val-Leu-Leu
145	Val-Leu-Leu-Ala
073	Leu-Leu
108	Leu-Leu-Ala
139	Leu-Leu-Ala-Ala
173	Leu-Leu-Ala-Ala-Val
053	Leu-Ala
093	Leu-Ala-Ala
027	Ala-Ala
086	Ala-Ala-Val
171	Ala-Ala-Val-Leu-Leu
	Ala-Val-Leu-Leu-Ala

The mass spectra of two crucial tripeptide derivatives are shown in Figures 3 and 4. They represent scans 103 and 109 of the experiment in Figure 2. The sequence ions unambiguously established the structure of these peptides.

In addition to the dipeptide derivatives listed in Table V, small amounts of the polyamino alcohols derived from the corresponding inverted dipeptides were also detected. For example, in the GC peak for Leu-Ala, some Ala-Leu was found. The relative abundance of this inverted dipeptide varied but never reached more than a few percent of the normal dipeptide. This interconversion has not been observed in the derivatization of synthetic dipeptides and is presumably due to transpeptidization of the cyclic peptide malformin C via diketopiperazines formed in the acid hydrolysis. The unusual hydrolysis conditions (glacial acetic acid–HCl instead of the normally employed 6 N aqueous HCl) may also encourage diketopiperazine formation, as is observed with more dilute aqueous acid.[8]

The evidence discussed thus far limits the structural possibilities for malformin C to **2** and **3**. To determine the sequences of leucines the stereochemistry of the dipeptide Val-Leu had to be ascertained. Since valine was known to have the L configuration the dipeptide had to be either L-Val-L-Leu or L-



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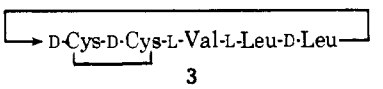

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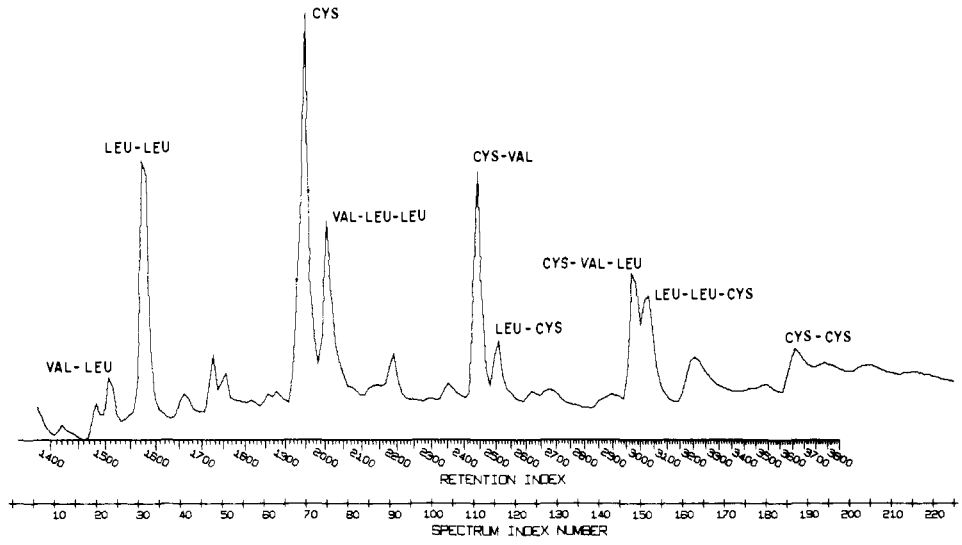

Figure 1. Total ionization plot (gas chromatogram) of *O*-trimethylsilylated polyamino alcohols obtained by derivatization of an acid hydrolysate [AcOH, concentrated HCl (4:1), 100°, 1 h] of dibenzylmalformein C.

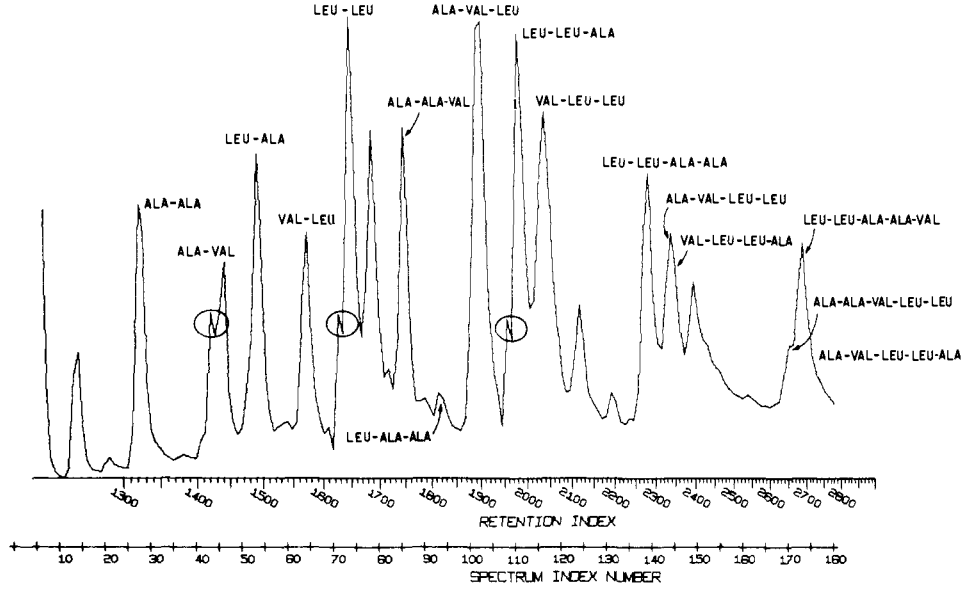

Figure 2. Total ionization plot of *O*-trimethylsilyated polyamino alcohols obtained by derivatization of an acid hydrolysate [AcOH, concentrated HCl (4:1), 100°, 1.25 h] of desthiomalformin C. Circles indicate apparent doublets due to a temporary reduction of the electron multiplier gain to reduce the signal for the three major components.

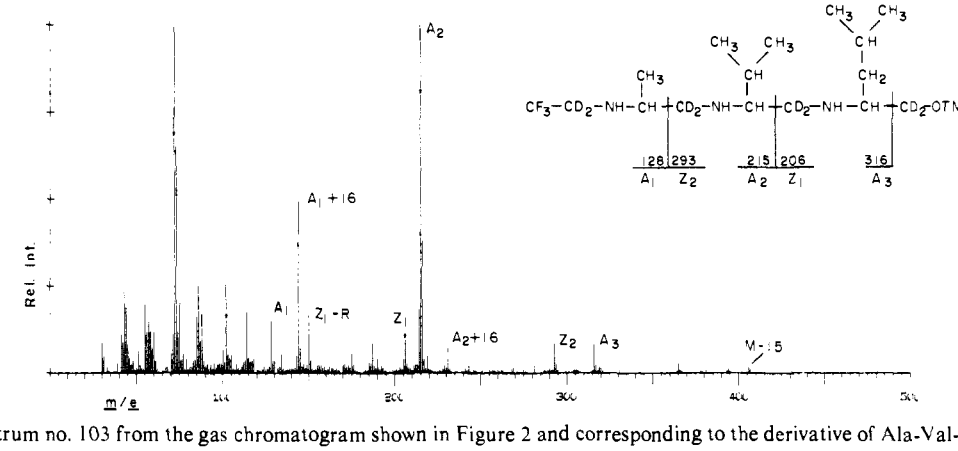

Figure 3. Mass spectrum no. 103 from the gas chromatogram shown in Figure 2 and corresponding to the derivative of Ala-Val-Leu.

Val-D-Leu. Authentic samples of these were prepared and the *N*-trifluoroacetylated peptide methyl esters were chromatographed on a glass capillary column (100 m × 0.75 mm internal diameter wall coated with Silanox 101 and SE-30).[9] The diastereomers were clearly resolved (Figure 5a), the L-Val-L-Leu isomer eluting first as is generally observed (Figure 5b).[10,11] A sample of desthiomalformin C was hydrolyzed partially; the resulting mixture of peptides was methylated,

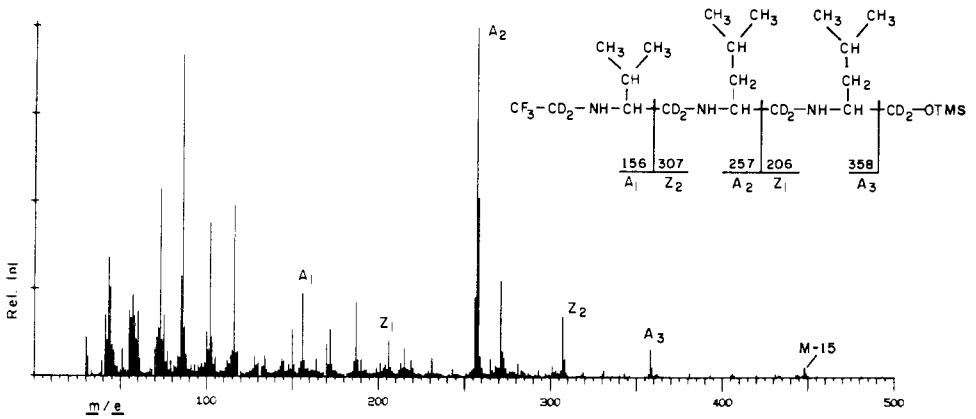

Figure 4. Mass spectrum no. 109 from the gas chromatogram shown in Figure 2 and corresponding to the derivative of Leu-Leu-Ala.

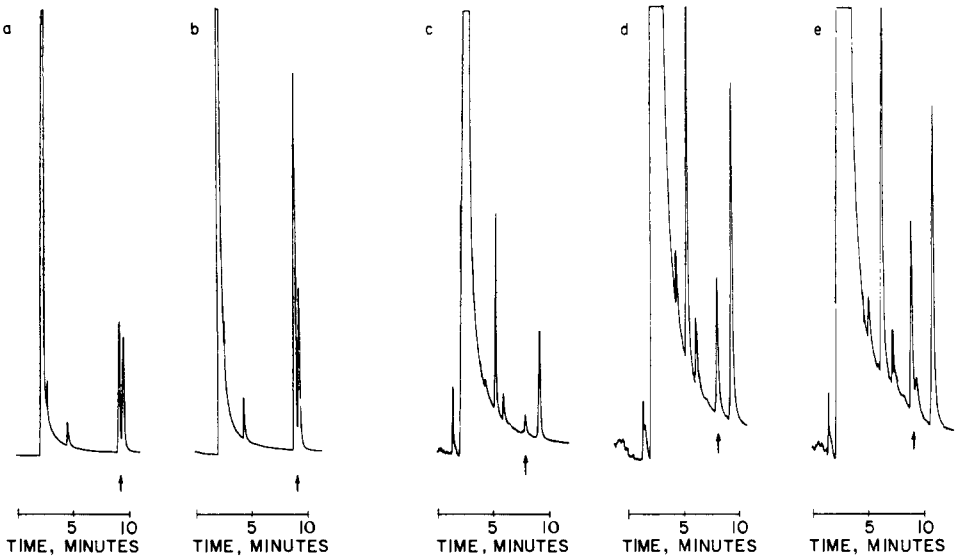

Figure 5. (a) Gas chromatogram of TFA-L-Val-DL-Leu-OMe. (b) Gas chromatogram of TFA-L-Val-DL-Leu-OMe with TFA-L-Val-L-Leu-OMe coinjected. (c) Partial acid hydrolysate of desthiomalformin C, TFA-peptide methyl esters. (d) Same as C with TFA-L-Val-D-Leu-OMe coinjected. (e) Same as C with TFA-L-Val-L-Leu-OMe coinjected. Since assignments were verified by mass spectra, no attempt was made to exactly reproduce gas chromatographic conditions.

N-trifluoroacetylated, and chromatographed. The fraction emerging after approximately 8 min (Figure 5c) was identified as TFA-Val-Leu-OMe by its mass spectrum. Coinjection of the authentic L-Val-D-Leu derivative with the peptide derived mixture produced a single peak in the gas chromatogram (Figure 5d), while coinjection of the L-Val-L-Leu standard with the "peptide mixture" led to a doublet (Figure 5e) of approximately the same spacing as that produced by L-Val-DL-Leu (Figure 5a,e). This means that Val-Leu in malformin C is L-Val-D-Leu and malformin C itself must have structure **2**.

The lethal potency of malformin C was determined in newborn and 28-day-old Sprague–Dawley rats. The compound was dissolved in Me_2SO at concentrations which permitted injection of 2 μl/g of body weight. Each animal received a single intraperitoneal injection when it was less than 24 h or 28 days old; controls received Me_2SO alone. Mortality was recorded for a period of 7 days and LD_{50} values were calculated from the dose-response regression line. The LD_{50} value for newborn rats was 0.90 mg/kg of body weight (95% confidence interval 0.69–1.19); comparable values for 28-day-old animals were 0.87 mg/kg (0.28–2.70). The mechanisms of toxicity are under investigation.[12]

Malformin C showed antibacterial activity against *Bacillus subtilis* on an agar (1.5%) medium containing 5 g of glucose, 1 g of sodium citrate, 14 g of KH_2PO_4, 6 g of K_2HPO_4, 0.25 g of $MgSO_4$, and 2 g of $(NH_4)_2SO_4$ per liter.[13]

The biological activity of malformin C was compared with that of malformin A.[14] In the corn root curvature assay the optimum concentration for both was 0.1 μg/ml. When applied to the apical bud of *Phaseolus vulgaris* seedlings, malformin C induced growth abnormalities which were identical with those induced by malformin A.[15]

Experimental Section

All reactions were performed under a nitrogen atmosphere and solvents were evaporated on a rotary evaporator under vacuum. Melting points were taken on a Kofler hot-stage microscope and are corrected. Optical rotations were determined on a Perkin-Elmer 141 polarimeter. Ir spectra were determined on a Perkin-Elmer 247 spectrophotometer. NMR spectra were recorded on a Perkin-Elmer R-22 90-MHz or on a Varian Associates 60-MHz instrument. Chemical shift values are reported in parts per million relative to Me_4Si as internal standard. Routine GC analyses were run on a Perkin-Elmer Model 990 gas chromatograph equipped with a flame ionization detector and a 3-ft glass column (⅛ in. i.d.) packed with 3% OV-17 on Gas Chrom Q (100–120 mesh). The temperature was programmed linearly from 60 to 330 °C at 12° per minute. Helium was the carrier gas flowing at 30 ml/min. GC–MS runs were done on a Perkin-Elmer Model 990 GC coupled with a glass frit to a continuously scanning Hitachi RMU-6L mass spectrometer which is interfaced with an IBM 1800 computer for data acquisition and processing. The system has been described elsewhere.[16] The high-resolution mass spectra were taken on a CEC21-110B mass spectrograph using an ion source temperature of 250°, an ionization potential of 70 eV, and an ionizing current of 200 μA. The samples were intro-

duced through a vacuum lock directly into the ion source. A photographic plate was used to record the spectra. The glass capillary column was 100 m × 1.3 mm o.d., 0.75 mm i.d., and was wall coated with Silanox 101 (Cabot Corp., Boston, Mass.) and SE-30 (GC Grade, Applied Science Laboratories, Inc., State College, Pa.). It was used in a Perkin-Elmer Model 990 gas chromatograph with a flow rate of 5 ml/min (He) and a scavenger gas flow of 25 ml/min (He). No splitter was used and samples (0-1.5 μl) were injected directly onto the column. The temperature was linearly programmed from 150 to 250° at 1.5°/min.

Authentic L-Val-L-Leu was purchased from Sigma Chemical Corp., St. Louis, Mo., while L-Val-D-Leu and L-Val-DL-Leu were prepared in these laboratories.

Production of Malformin C. A blake bottle containing a layer of Czapek's solution agar, enriched with 0.5% yeast extract and 0.5% casamino acid, was inoculated with spores from a soiltube and incubated at 30°. After sufficient sporulation the culture was used for fermentation. For the fermentation 10-20 Fernbach flasks (2.8 l., wide neck) were filled with white wheat (300 g) and warm water (150 ml). The spore inoculum was prepared by washing the spores off the blake bottle with 50-100 ml of 0.01% sodium lauryl sulfate. Each Fernbach flask was inoculated with spore suspension (10 ml) and kept on the shaker at 200 rpm, 10° for 10-12 days. The grains were extracted with CH_2Cl_2 (2 × 500 ml per flask) overnight. The mixture was filtered through glass wool and the filtrate homogenized for 60 s with additional CH_2Cl_2 (500 ml). The homogenate was filtered through cheesecloth, dried ($MgSO_4$), and filtered through Celite. The filtrate was evaporated and the oily residue poured slowly into ice-cold petroleum ether. After standing at 0° overnight, the precipitate (petroleum ether insoluble fraction) was filtered and dried in vacuo.

Isolation of Malformin C. A solution of this precipitate (2.5 g) in CH_2Cl_2 (300 ml) was extracted with 2 N Na_2CO_3 (150 ml). Evaporation of solvent from the dried ($MgSO_4$) organic layer left a red glass (1.67 g). The glass was dissolved in EtOAc (50 ml), the solution filtered from a small amount of insoluble material, and the filtrate chromatographed on a column of SiO_2 (70-230 mesh, EM Reagents, 200 g, 3.2 × 58.5 cm, flow rate 55 drops/min), eluting with EtOAc. Fractions (17 ml) were collected, and fractions 17-28 were combined and evaporated, and the residue was triturated with acetone (10 ml). The suspension was left standing at room temperature overnight before filtration of the light yellow colored powder (254 mg), which was washed with acetone (10 ml). The filtrate was concentrated to ca. 3 ml and left standing at room temperature for several days prior to filtration of a light yellow colored powder (139 mg). The combined yield of solids was dissolved in a mixture of hot EtOAc and EtOH (3:1) and chromatographed on a column of alumina (2.8 × 21 cm, Baker, 100 g), eluting with a mixture of EtOAc and EtOH (3:1). The eluent volume, 0-300 ml, was evaporated, leaving a colorless powder (369 mg, 15%): mp >300° dec; $[\alpha]^{25}D$ −37.4° (*c* 1.01, Me_2SO), $[\alpha]^{25}D$ −37.4° (*c* 0.52, methyl cellosolve); ir (KBr) 3280, 2930, 1630, 1500 cm^{-1}; NMR (Me_2SO-d_6) δ 8.72 (d, 1 H, *J* = 5 Hz, exchangeable with D_2O), 8.42 (d, 1 H, *J* = 6 Hz, exchangeable with D_2O), 7.73 (d, 1 H, *J* = 8 Hz, exchangeable with D_2O), 7.28 (d, 1 H, *J* = 9 Hz, exchangeable with D_2O), 7.06 (d, 1 H, *J* = 11 Hz, exchangeable with D_2O), 4.80-3.69 (m, 5 H), 3.46 (d, 1 H, *J* = 15 Hz), 3.17 (m, 3 H), 1.98 (m, 1 H), 1.78-1.00 (m, 6 H), 0.86 (m, 18 H); *m/e* (found) 529.2390, calcd for $C_{23}H_{39}N_5O_5S_2$ 529.2393.

Dibenzylmalformein C. To a suspension of malformin C (53 mg, 0.1 mmol) in redistilled liquid NH_3 was added dithiothreitol (309 mg, 2 mmol). The solution was kept at the boiling point for 1 h before freshly distilled benzyl chloride (4.7 ml, 5.06 g, 40 mmol) was added. After 10 min the NH_3 was allowed to evaporate. The last traces of liquid were removed by evaporation at 0.1 mm, leaving a white, amorphous, solid residue. The solid was triturated with MeOH (2 × 5 ml) and Et_2O (2 ml), yielding a colorless, amorphous solid (42.5 mg, 60%): mp >300°; $[\alpha]^{25}D$ +23.8° (*c* 1.27, Me_2SO), $[\alpha]^{25}D$ +45.9° (*c* 0.73, CF_3COOH); ir (KBr) 3280, 2950, 1625, 1535, 685 cm^{-1}; NMR δ 9.03-8.29 (m, 3 H), 7.83-7.26 (m, 12 H), 4.67-3.87 (m, 5 H), 3.70 (s, 4 H), 2.77 (m, 4 H), 2.00 (m, 1 H), 1.43 (m, 6 H), 0.88 (m, 18 H); *m/e* (found) 711.3461, calcd for $C_{37}H_{53}N_5O_5S_2$ 711.3488.

Desthiomalformin C. Malformin C (582.6 mg, 1.1 mmol) was dissolved in hot methyl cellosolve (700 ml), and W-2 Raney nickel (16 ml of settled material in EtOH, ca. 10 g, total volume 35 ml) was added. The suspension was heated at reflux for 1.5 h. The mixture was filtered hot, once through Celite on filter paper and once through Celite on a glass frit. The solvent was evaporated and the residue dried for 2 h at 81° (0.005 mm), yielding a colorless powder (408.8 mg, 79%): mp >300°; ir (KBr) 3280, 2950, 1635, 1540 cm^{-1}; *m/e* (found) 467.3121, calcd for $C_{23}H_{41}N_5O_5$ 467.3108.

Hydrolysis of Desthiomalformin C and Isolation of Amino Acid Hydrochlorides. Desthiomalformin C (300 mg, 0.64 mmol) was dissolved in AcOH (30 ml) with warming, and the resulting solution was diluted with concentrated HCl (30 ml). The solution was heated in a sealed tube at 110° for 22 h and the mixture was then evaporated and excess HCl was removed from the residue by addition and evaporation of water (3 × 10 ml). The residue was dried at room temperature (2 μ) for 8 h, yielding a colorless solid (473 mg) which was chromatographed on a cellulose column (125 g, 2.9 × 54 cm), eluting with a 1-butanol-AcOH-water (4:1:5) mixture. The flow rate was 0.36-0.30 ml/min. Fractions (3.6-3.0 ml) were collected and amino acids detected by TLC on cellulose plates (Analtech, Avicel, 250 μ), using the organic phase of a 1-butanol-AcOH-water (50:1:50) mixture as solvent system. Fractions containing separated amino acids were evaporated on a rotary evaporator followed by 1 h at room temperature (5 μ). To the residues was added 6 N HCl (10 ml), followed by evaporation on a rotary evaporator and 8 h at room temperature (5 μ). The residues were stored over $CaSO_4$ and NaOH in a vacuum desiccator overnight. Leucine was found in fractions 67-80, from which the HCl salt (182.3 mg) was isolated. Fractions 81-86 contained a mixture of leucine and valine. Fractions 87-99 contained valine, from which valine HCl (60.6 mg) was isolated. Alanine HCl (156.2 mg) was isolated from fractions 108-161. The residues were dissolved in water (10 ml), decolorized with charcoal, and filtered through a glass frit, and the charcoal was washed with water (10 ml). Evaporation of the clear, colorless filtrates resulted in colorless HCl salts which were dried at room temperature (5 μ) for 5 h and then used for determination of optical rotations.

Partial Acid Hydrolysis. Because of their low solubility, even in 6 N HCl, the *S*-benzyl and desthio derivatives of malformin C were hydrolyzed in a mixture of glacial acetic and hydrochloric acid. Typically, 4 mg of desthiomalformin C was dissolved in 2.5 ml of AcOH-concentrated HCl (4:1). The solution was degassed, sealed in an evacuated hydrolysis tube, heated to 100 °C for 75 min, and lyophilyzed to yield a fluffy white powder.

Conversion to Polyamino Alcohols. The derivatization steps for conversion of the acid hydrolyzate into a mixture of trifluorodideuteriopolyamino alcohols have been described elsewhere.[7] Methylation was done in 3 ml of freshly prepared methanolic HCl at room temperature for 30 min. Trifluoroacetylation was carried out with 3 ml of methyl trifluoroacetate in methanol (1:2). The trifluoroacetylpeptide methyl esters were reduced in 3 ml of 0.2 M lithium aluminum deuteride (LiAlD) in glyme for about 48 h, the temperature being gradually raised from 0 to 90° where it remained for the final 24 h. The reduction was quenched by dropwise addition of methanol and the volume was brought up to 10 ml and the aluminum salts were precipitated with 12 drops of water. The slurry was filtered and the precipitate was washed with 2 × 6 ml of fresh methanol. The filtrate was evaporated to dryness, and the residue was taken up in 3 × 6 ml of chloroform and refiltered. The solvent was removed, and the polyamino alcohols were silylated in 200 μl of trimethylsilyldiethylamine (Me_3SiDEA)-pyridine (1:1) at 55 °C for 1 h. About 2% of the silylation mixture was injected onto the gas chromatograph.

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(13) The antibacterial activity of malformin C was kindly measured by Professor A. L. Demain and Miss B. Kobbe, Department of Nutrition and Food Science, MIT.
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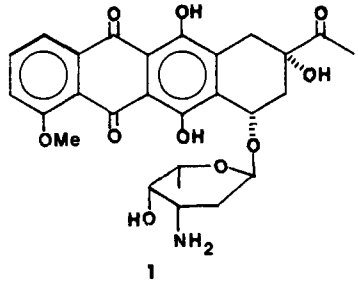
Communications to the Editor

A ^{13}C Nuclear Magnetic Resonance Study of the Biosynthesis of Daunomycin from $^{13}CH_3{}^{13}CO_2Na$

Sir:

A number of plausible connectivity patterns are indicated (Scheme I) for the biosynthesis of the polyketide antibiotic daunomycin (**1**) via an acetate–polymalonate route (Scheme I-c or d) or a propionate–polymalonate pathway (Scheme Ia or b). We have applied the Tanabe technique[1] to this biosynthetic problem and have obtained results that are consistent with only route a: a propionate "starter" and nine successive malonate condensations with loss of the terminal carboxyl.

Streptomyces peucetius (ATCC no. 21354) was maintained on malt extract agar[2a] and grown in a dry yeast/glucose production medium under submerged conditions in shake flasks. After considerable experimentation yields of **1** on the order of



1

15 μg/ml could be isolated. For carbon NMR (^{13}C NMR) studies crude **1** was subjected to hydrolysis and acetylation with formation of daunomycinone tetracetate (**2**).

Through the use of the $Cr(acac)_3$ T_1 suppresor technique[3,4] peaks for all carbons are observed and are of comparable intensity in the ^{13}C NMR spectrum of **2** (Figure 1a). Application of chemical shift data from comparable anthraquinones,[3] substituent shift calculations,[5] and single frequency and off-resonance decoupling experiments allowed, with one or two ambiguities in the 135 ppm region, assignment of all peaks. These assignments are presented in Table I.

Scheme I

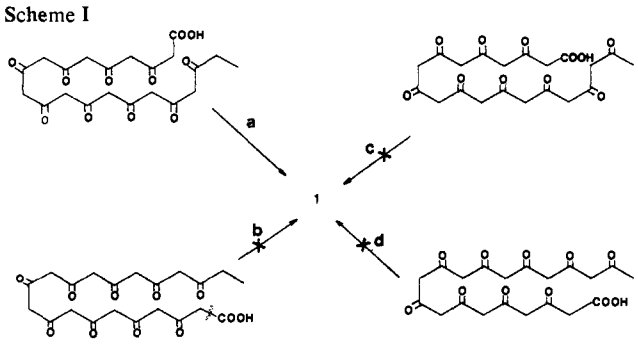
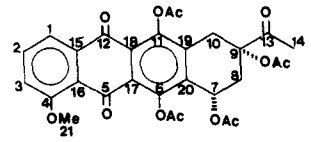


Table I. Chemical Shift Assignments for Carbons of Daunomycinone Tetraacetate (tabulates by the numbering system shown).



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Carbon	Chemical shift[a]	Carbon	Chemical shift
C-1	119.3[b]	C-13	205.0
C-2	134.1[b]	C-14[b]	24.0
C-3	118.3[b]	C-15	135.8
C-4	159.7	C-16[h]	122.3
C-5	180.6[d]	C-17[c]	126.2
C-6	146.7[c]	C-18[c]	125.2
C-7	62.1[b]	C-19[c]	134.7
C-8	30.9[b]	C-20[c]	134.2
C-9	80.4[e]	C-21[b]	57.0
C-19	31.3[b]	acetate methyls	20.8–21.0
C-11	145.1[c]	acetate carbonyls	168.9–170.7
C-12	182.0[d]		

[a] Chemical shifts were measured in $CDCl_3$ in parts per million relative to internal Me_4Si. [b] This carbon was positively assigned on the basis of single frequency decoupling experiments. [c] The C-11, C-6; C-17, C-18; and C-19, C-20 pairs were distinguished primarily by the single labeled acetate experiments. [d] C-5 and C-12 were distinguished by the single-labeled acetate experiments, but a comparison of this compound with islandicin triacetate would predict the given chemical shifts. [e] Sharp singlet in off-resonance decoupling experiments.

The incorporation experiments with ^{13}C-labeled sodium acetate (C_1, C_2, $C_{1,2}$ all 91% isotopic purity) entailed culturing *S. peucetius* in the dry yeast/glucose medium for 5 days and pulsing the growing organism twice daily on the second, third, and fourth days with 50 mg of the labeled acetate. The results of these experiments are presented in Table II, summarized in Figure 2, and discussed below.

CH_3 $^{13}CO_2Na$: Growth of *S. peucetius* as above in the presence of sodium [1-^{13}C]acetate afforded **2**, the ^{13}C NMR spectrum of which showed appreciable incorporation of ^{13}C at carbons 2, 4, 5, 6, 7, 15, 18, and 19.